In vitro and *in vivo* characteristics of stem cells from human exfoliated deciduous teeth obtained by enzymatic disaggregation and outgrowth

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Directed by Professor Seong-Oh Kim

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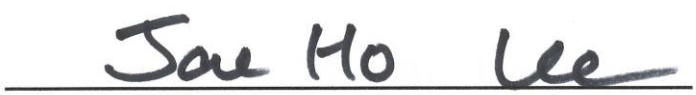
This certifies that the dissertation of Mijeong Jeon is approved.

Thesis Supervisor: Seong-Oh Kim



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감사의 글

박사학위 논문이 완성되기까지 물심 양면으로 도와주시고 지도해주신 분들에게 감사의 마음을 전하고 싶습니다.

학위과정을 제안해주시고 박사학위 지도교수님이 되어주시고 아낌없는 격려와 많은 도움을 주신 김성오 교수님, 인생을 살아가는데 필요한 조언과 격려를 해주신 이제호 교수님, 술자리가 무서워서 많이 피했지만 관심과 격려를 주신 최형준 교수님, 연구의 방향을 잡아주시고 연구가 완성될 수 있도록 도와 주신 송제선 교수님, 소아치과에서 있었던 4년 동안 따뜻한 미소로 맞아주신 존재만으로도 큰 힘이 되었던 손흥규 교수님, 보이지 않는 곳에서 챙겨주신 최병재 교수님, 제가 진심으로 행복하길 바란다고 말씀해주신 이효설 교수님께 깊은 감사를 드립니다. 또한 학위 과정 내내 박사학위를 받을 수 있게 도와주시고 좋은 논문이 나올 수 있도록 끝까지 지도해 주시고 조언해주신 신동민 교수님, 정한성 교수님께 감사드립니다.

바쁜 병원 생활 속에서도 관심과 도움을 준 소아치과 모든 선생님들, 연구가 힘들 때마다 함께 고민해주고 도와주는 정재은 선생님과 삭막한 연구실에 큰 웃음과 활력을 준 코디네이터 이다선 선생님, 임경은 비서에게도 감사를 드립니다. 같이 수업 듣고 친해져서 안부도 묻고 고민도 함께 나누고 서로서로 챙겨준 혜진이와 지은이에게도 너무너무 고맙습니다. 한 분씩 다 나열하지 못했지만 도움을 준 모든 분들께 진심으로 감사드립니다.

마지막으로 항상 옆에서 응원해주시고 힘을 주시는 늘 제가 최고라고 말씀해주시는 사랑하는 부모님과 하나뿐인 동생에게 고마움과 사랑의 마음을 전합니다.

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In vitro and *in vivo* characteristics of stem cells from human exfoliated deciduous teeth obtained by enzymatic disaggregation and outgrowth

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(Directed by Professor Seong-Oh Kim)

Stem cells from human exfoliated deciduous teeth (SHED) are a good source of dental tissue for regeneration therapy, and can be obtained using different primary culture methods. The aim of this study was to determine the differences in the *in vitro* and *in vivo* characteristics between SHED isolated via enzymatic disaggregation (e-SHED) and outgrowth (o-SHED) primary culture methods.

Dental pulp stem cells were isolated from exfoliated deciduous teeth by enzymatic disaggregation (*n*=7) and outgrowth (*n*=7). Their proliferation potential and colony-forming ability were evaluated *in vitro*, as was their mesenchymal stem-cell-marker expression, and their differentiation was verified using quantitative real-time PCR (qPCR) and histochemical staining. In addition, the qualitative and quantitative characteristics of the hard tissue that was generated after *in vivo* transplantation were compared using HE staining, immunohistochemical staining, qPCR, and alkaline phosphatase analysis.

The cell-proliferation potential, colony-forming ability, and Stro-1 and CD146 expression were higher in e-SHED. While the *in vitro* adipogenic differentiation potential was greater in e-SHED, the *in vitro* osteogenic differentiation did not differ significantly between the two cell types. Although *in vivo* hard tissue formation was greater following transplantation of o-SHED into mice, there was no difference in the quality of hard tissue generated by e-SHED and o-SHED.

The findings of this study indicate that e-SHED exhibit stronger stemness characteristics, but that o-SHED are more suitable for hard-tissue regeneration therapy in teeth.

Keywords: Stem cells from human exfoliated deciduous teeth (SHED), enzymatic disaggregation, outgrowth, *in vivo* transplantation, hard tissues

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I. Introduction

Teeth stem cells are obtained from oral niches that, like stem cells, vary hugely; the niche plays an essential role in the maintenance, proliferation, and differentiation of stem cells. Stem cells interact with the niche and are influenced by its characteristics,

such as its spatial arrangement and the signals it secretes (Hardy et al., 1979; Brinster and Zimmermann, 1994; Gonczy and DiNardo, 1996; Xie and Spradling, 2000; Kiger et al., 2001; Jones and Wagers, 2008). Stem cells start to differentiate when they are distant from the niche or when they no longer receive the signals from the niche that maintain their stem cell characteristics (Hardy et al., 1979; Gonczy and DiNardo, 1996; Jones and Wagers, 2008). Mesenchymal stem cells obtained from teeth include dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHED), stem cells from the apical papilla, dental follicle progenitor cells, and periodontal ligament stem cells (PDLSCs) (Estrela et al., 2011; Rodriguez-Lozano et al., 2011). In deciduous teeth specifically, stem cells are found in human exfoliated deciduous teeth (Miura et al., 2003), and it has recently been shown that these teeth also contain PDLSC (Silverio et al., 2010; Song et al., 2012). These mesenchymal stem cells are advancing the research into tooth regeneration (Gronthos et al., 2000; Huang et al., 2010; Guo et al., 2012; Zhu et al., 2012; Ji et al., 2013; Wang et al., 2013).

SHED are particularly attractive as a source of stem cells since they can be harvested noninvasively, unlike bone marrow and adipose tissue, and they have a higher proliferative rate than other stem cells (Miura et al., 2003; Seo et al., 2008; Rodriguez-Lozano et al., 2011; Wang et al., 2012). Moreover, SHED can differentiate into osteoblasts, adipocytes, and neural cells (Miura et al., 2003; Seo et al., 2008; Nourbakhsh et al., 2011). SHED can produce hard tissue in transplanted mice, and are able to differentiate into dentate gyrus cells in the brains of transplanted mice

(Miura et al., 2003). It has also been reported that SHED can form hard tissues in the calvarial defect model, and that compared to DPSCs they have superior colony-forming ability and adipogenic and osteogenic differentiation potentials (Seo et al., 2008; Wang et al., 2012).

Several primary culture methods to obtain stem cells: fine dissection, mechanical disaggregation, and enzymatic disaggregation (Kerkis et al., 2006; Freshney, 2010). Fine dissection involves explant and outgrowth methods using small pieces of chopped tissue, mechanical disaggregation requires the rapid formation of a cell suspension using a sieve, syringe, and pipette, and enzymatic disaggregation utilizes cold or warm trypsin, collagenase, and dispase to separate the cells from their tissues of origin (Kerkis et al., 2006; Freshney, 2010).

Previous studies have isolated multipotent stem cells from teeth using two methods: enzymatic disaggregation and outgrowth (Bakopoulou et al., 2011; Tanaka et al., 2011; Karamzadeh et al., 2012; Hilkens et al., 2013). Deciduous teeth stem cells isolated by enzymatic disaggregation were found to be superior to those isolated using the outgrowth method, as assessed by measuring the expression of mesenchymal stem-cell markers and the osteogenic differentiation potential (Bakopoulou et al., 2011). However, most of these studies have only evaluated the *in vitro* characteristics of the isolated stem cells; few studies have compared their *in vivo* characteristics when using different isolation methods.

Therefore, in the present study stem cells were isolated from human exfoliated deciduous teeth using two isolation methods: enzymatic disaggregation and

outgrowth. The proliferation, expression of stem-cell markers, and *in vitro* adipogenic and osteogenic differentiation of the stem cells were confirmed. The hard tissue that was produced by the stem cells after *in vivo* transplantation was analyzed qualitatively and quantitatively using the quantitative polymerase chain reaction (qPCR), alkaline phosphatase (ALP) assay, hematoxylin and eosin staining (HE), and immunohistochemistry (IHC).

II. Materials and Methods

1. Cell Culture

The SHED were obtained from the deciduous teeth of 14 children (aged 5–11 years; 9 males and 5 females) under approved guidelines set by the Institutional Review Board of the Dental hospital, Yonsei University (approval no. #IRB 2-2012-0003). The deciduous anterior teeth used in this study were near to natural exfoliation, with less than one-third of the root remaining, and without any deep caries, restoration, periapical lesions, or internal resorption. After extraction, the deciduous teeth were divided randomly in a single-blinded manner into two groups according to the stem cell isolation method: (1) enzymatic disaggregation (n=7) and (2) outgrowth (n=7). Pulp tissue from the deciduous teeth was extirpated using a barbed broach (Mani, Utsunomiya Toshi-ken, Japan), washed with PBS (Invitrogen, Carlsbad, CA, USA), and subjected to primary culture using enzymatic disaggregation and outgrowth methods. In the enzymatic disaggregation group, the pulp tissue was treated with collagenase type I (3 mg/ml, Invitrogen) and dispase (4 mg/ml, Invitrogen) for 30 min at 37℃ and then were filtered through a 70-μm cell strainer. The SHED were cultured in a cell culture medium comprising alpha-minimum essential medium (α-MEM; Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), and 10 mM L-ascorbic acid (Sigma-Aldrich, St Louis, MO, USA) at 37℃ in 5% CO_2. In the outgrowth group, the pulp tissue was separated from the teeth and cultured using the outgrowth method as described in Song, et al (Song et al., 2010). In brief, the pulp tissue was cut perpendicularly into three fragments that were

subsequently placed onto a 60-mm culture dish (BD Falcon, Lincoln Park, NJ, USA), and covered with a cover glass to allow the cells to grow out. The cells were cultured in the culture medium described above at 37℃ in 5% CO_2.

Ultimately, SHED were obtained from seven different donors by enzymatic disaggregation (hereafter referred to as e-SHED), and from seven different donors by outgrowth (hereafter referred to as o-SHED). The isolated stem cells originated from at least four different donors from each group were combined at passage 2, and cells at passages 3–5 were used for further experiments.

2. Proliferation Assay

Cell proliferation was performed as described in Song, et al (Song et al., 2012). In brief, the SHED were plated into 24-well culture plates (BD Falcon) at a density of 5000 cells/well. On alternate days (i.e., days 1, 3, 5, 7, and 9), the quantity of water-soluble colored formazan from the Cell Counting Kit (CCK)-8 assay (Dojindo Laboratories, Kumamoto, Japan) formed by the activity of dehydrogenases in living cells was measured using a spectrophotometer (Benchmark Plus microplate spectrophotometer, Bio-Rad Laboratories Inc., Hercules, CA, USA) at 450 nm. Proliferation assay data were obtained from three independent experiments, with all samples run in triplicate.

3. Colony-Forming-Unit–Fibroblast Assay

The colony-forming-unit–fibroblast (CFU-F) assay was conducted as described in Song et al (Song et al., 2012). Briefly, the SHED were seeded into 6-well culture plates (BC

Falcon) at a low concentration of 480 cells/well and incubated for 9 days. They were fixed with 10% buffered formalin (Sigma-Aldrich) for 1 hour, and stained with 0.3% crystal violet (BD Biosciences, San Jose, CA, USA) for 5 min. The number of colonies containing more than 50 cells was counted with the aid of a light microscope (BS40, Olympus, Tokyo, Japan). Data were obtained from three independent experiments.

4. Flow Cytometry Analysis

The method of flow cytometry analysis is described in a previous study (Song et al., 2012). In brief, following detachment of the SHED using 0.2% ethylenediaminetetraacetic acid (EDTA; Fisher Scientific, Houston, TX, USA) in PBS (Invitrogen), the cells were resuspended in flow cytometry staining buffer (eBiosciences, San Diego, CA, USA). The cells were then incubated with an appropriate amount of mouse monoclonal antihuman antibodies [fluorescein-isothiocyanate-conjugated CD146, F-phycoerythrin (PE)-conjugated CD90, PE-conjugated CD105, and PE-conjugated CD31; all supplied by eBiosciences], as per the manufacturer's instructions. For antihuman Stro-1 staining, 5 μg of the antihuman Stro-1 primary antibody (IgM, R&D Systems, Minneapolis, MN, USA) was applied per 1×106 cells for 1 hour, and followed by the secondary antibody [PE-conjugated goat antimouse antibody, 0.1 μg/1×10^6 cells; IgM, SouthernBiotech, Birmingham, AL, USA)] for 30 min. The control conditions involved omitting either the primary antibody or the fluorescent dye-conjugated antibodies. All procedures were performed in the dark at 4℃. The expression profiles were examined using a flow cytometer (FACSCalibur, BD Biosciences, Franklin Lakes,

NJ, USA) and analyzed using FCSExpress V4 software (De Novo Software, Los Angeles, CA, USA). Expression was defined as being positive with the level of fluorescence exceeded 99% of the corresponding control. Data were obtained from three independent experiments.

5. Adipogenic and Osteogenic Differentiation

The differentiation methods used are described in Song, et al (Song et al., 2012). In brief, for adipogenic differentiation, the cells were seeded into 12-well culture plates (BD Falcon) at a density of 1×10^4 cells/well. Upon reaching 90% confluence, the cells were treated for 2 weeks with adipogenic induction medium [α-MEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μM dexamethasone (Sigma-Aldrich), 10 μg/ml human insulin (Sigma-Aldrich), 100 μM indomethacin (Sigma-Aldrich), and 500 μM 3-isobutyl-L-methylxanthine (Sigma-Aldrich)]. The medium was then exchanged for adipogenic maintenance medium (α-MEM containing 10% FBS, 1% antibiotics, and 10 μg/ml human insulin) for a further week. A negative control was produced by cultured cells in growth medium only. The intracellular accumulation of lipids was visualized after the 3-week differentiation procedure using Oil Red O staining. Briefly, the cells were fixed for 30 min with 10% buffered formalin (Sigma-Aldrich) at 4℃ and stained with 0.2% Oil Red O (Sigma-Aldrich) for 10 min at room temperature. Data were obtained from five independent experiments.

For osteogenic differentiation, the cells were prepared in 12-well culture dishes as described for adipogenic differentiation. Upon reaching 90% confluence, the cells were

treated with osteogenic induction medium [α-MEM containing 10% FBS, 1% antibiotics, 0.1 μM dexamethasone, 2 mM β-glycerophosphate (Sigma-Aldrich), and 50 μM ascorbic acid 2-phosphate] for 4 weeks. A control was produced by culturing cells in growth medium only. At the end of the 4-week differentiation period, mineralization nodules were visualized using Alizarin Red S staining and quantified with the aid of cetylpyridinium chloride. Briefly, cells were fixed as for adipogenic differentiation and then stained with 2% Alizarin Red S (pH 4.2; Sigma-Aldrich) for 20 min at room temperature. The Alizarin Red S dye was extracted using 10% cetylpyridinium chloride (Sigma-Aldrich). The absorbance at 570 nm was measured. Data were obtained from six independent experiments.

Relative changes in the expressions of genes encoding peroxisome proliferator-activated receptor γ2 (*PPARγ2*) and lipoprotein lipase (*LPL*) after adipogenic differentiation were evaluated by the quantitative reverse-transcription polymerase chain reaction (RT-PCR), and in those encoding alkaline phosphatase (*ALP*) and bone sialoprotein (*BSP*) for osteogenic differentiation, as described above. Adipogenic and osteogenic differentiation data were obtained from five and four independent experiments, respectively.

6. Quantitative Real-Time PCR

Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The integrity and concentration of the extracted RNA was evaluated using a spectrophotometer (NanoDrop ND-1000, Thermo

Scientific, Waltham, MA, USA). One-microgram aliquots of RNA were reverse transcribed to synthesize cDNA using a Maxime RT premix kit [oligo d(T)15 primer; Intron Biotechnology, Seongnam, Gyeonggi, Korea] according to the manufacturer's instructions. A real-time qPCR assay was performed with SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan) and a real-time PCR system (ABI 7300, Applied Biosystems, Carlsbad, CA, USA) as per the manufacturer's instructions. The qPCR conditions were 95℃ for 10 sec followed by 40 cycles of 95℃ for 5 sec and 60℃ for 30 sec, with a final 5-min extension at 72℃. Application specificity was confirmed by visualizing PCR products on 1.5% agarose gels and melting-curve analysis (from 60℃ to 95℃). The sequence and size of the primers are given in Table 1. The values for each gene were normalized to the expression levels of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and the relative expression levels of the studied genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

7. Transplantation

The transplantation procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Yonsei University (protocol no. #2012-0060). Cells were cultured in MEM culture medium until reaching 90% confluence. Approximately 3×10^6 cells were combined with 40 mg of macroporous biphasic calcium phosphate (MBCP; Biomatlante, Vigneux de Bretagne, France) and then transplanted into dorsal subcutaneous pockets (maximum four for each mouse) of male 5-week-old immunocompromised mice (n=10; BALB/c-nu, SLC, Shizuoka, Japan),

as described in previous studies (Kuznetsov et al., 1997; Song et al., 2012). Briefly, the mice were anesthetized using an intraabdominal injection of a combination of Zoletil (0.06 ml), Rompun (0.04 ml), and saline (0.9 ml) in a 0.3-ml volume into the right lower abdominal cavity. The experimental materials were then transplanted into subcutaneous pockets on the back of each mouse. MBCP particles placed alone constituted a negative control. All transplants were retrieved after 9 weeks and divided into two or three fragments for further analysis.

8. Histological Analysis of the Transplants

The transplant fragments (n=19 for e-SHED, n=20 for o-SHED) were fixed with 10% buffered formalin (Sigma-Aldrich) for 1 day and then decalcified with 10% EDTA (pH 7.4; Fisher Scientific Co.) for 3 weeks. The decalcified transplants were embedded in paraffin, sectioned at a thickness of 3 μm, and stained with HE. The hard-tissue-forming potential was evaluated by measuring the areas of newly formed hard tissue and of MBCP particles in each fragment using Image J software (version 1.45, NIH, Bethesda, MD, USA); the total area of the former was divided by the total area of the latter to give the proportion of newly formed bone in each fragment.

9. Immunohistochemistry Analysis of the Transplants

The sections were deparaffinized in xylene, rehydrated, and rinsed with distilled water. Protease K (Dako, Carpinteria, CA, USA) was used to retrieve the antigen for the osteocalcin (OC) staining, while no such treatment was performed for dentin sialoprotein

(DSP) staining. The sections were immersed in 3% hydrogen peroxide for 10 min to inactivate endogenous peroxidase activity and then incubated with primary antibody overnight. The primary antibodies were a 1:2500 dilution of antihuman OC (rabbit polyclonal antibody; #AB10911, Millipore, Temecula, CA, USA) and a 1:1500 dilution of antihuman DSP (rabbit polyclonal antibody; sc-33586, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Sections were subsequently incubated for 20 min with horseradish-peroxidase-labeled polymer conjugated with secondary rabbit antibody in an EnVision+ system kit (Dako). The color was developed using 3,3'-diaminobenzidine substrate (Dako) and counterstained with Gill's hematoxylin solution (Merck, Darmstadt, Germany). Negative control sections were stained in the same manner, but without the primary antibody reaction procedure.

10. qPCR in the Transplants

The relative expressions of the genes encoding dentin sialophosphoprotein (*DSPP*), runt-related protein 2 (*Runx2*), *OC*, osteopontin (*OPN*), and *GAPDH* were evaluated by qPCR. Immediately after retrieval, the transplant fragments (n=12 for e-SHED, n=18 for o-SHED) were homogenized using 0.5-mm-diameter stainless steel beads (Next Advance, Averill Park, NY, USA) and a bullet blender (Next Advance) in RLT buffer, which is a component of the RNeasy Mini Kit (Qiagen). Total RNA was isolated using the kit according to the manufacturer's instructions. The integrity and concentration of extracted RNA were evaluated using a spectrophotometer (NanoDrop ND-1000, Thermo Scientific). Aliquots (100 ng) of RNA were reverse transcribed to synthesize cDNA using

a Maxime RT premix kit [oligo d(T)15 primer, Intron Biotechnology] according to the manufacturer's instructions. A qPCR assay was performed using the aforementioned procedure. The sequences and sizes of the primers are given in Table 1. The expression level of each gene was calculated relative to that in the MBCP-alone transplants.

11. Quantitative Assay of ALP Levels in the Transplants

The level of ALP activity in each transplant was measured using Sensolyte p-nitrophenylphosphate (pNPP) ALP assay kit (AnaSpec, Fremont, CA, USA) according to the manufacturer's instructions. In brief, the retrieved transplant fragments (n=20 for e-SHED, n=20 for o-SHED) were rinsed and soaked in PBS (pH 7.4; Invitrogen) for 1 day. They were then lysed with Triton X-100 (provided in the assay kit), and pNPP was added to the supernatant of the lysates. ALP activity was determined by measuring the colorimetric change caused by dephosphorylation of pNPP (absorbance at 405 nm). The quantity of ALP was normalized against the total protein quantity in the supernatant of the same tissue lysate using a Thermo Scientific Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

12. Statistical Analysis

All experiments were performed at least in triplicate. The normality of the data was evaluated using the Shapiro-Wilk test ($p<0.05$). The Mann-Whitney U test ($p<0.05$) was performed for all experiments using SPSS software (19.0 SPSS, Chicago, IL, USA).

Table 1. qPCR forward (F) and reverse (R) primer sequences and sizes. The annealing procedures were performed at 60℃ for all primers.

Gene	Primer sequence (5'–3')	Size (bp)	Reference
DSPP	F: GGGATGTTGGCGATGCA R: CCAGCTACTTGAGGTCCATCTTC	70	(Wei et al., 2007)
Runx2	F: CACTGGCGCTGCAACAAGA R: CATTCCGGAGCTCAGCAGAATAA	127	(Qian et al., 2010)
OPN	F: ACCTGAACGCGCCTTCTG R: CATCCAGCTGACTCGTTTCATAA	66	(Dyson et al., 2007)
OC	F: CAAAGGTGCAGCCTTTGTGTC R: TCACAGTCCGGATTGAGCTCA	150	(Garlet et al., 2007)
BSP	F: CTGGCACAGGGTATACAGGGTTAG R: ACTGGTGCCGTTTATGCCTTG	182	(Fujii et al., 2008)
ALP	F: GGACCATTCCCACGTCTCAC R: CCTTGTAGCCAGGCCCATTG	137	(Tomokiyo et al., 2008)
PPARγ2	F: ACAGCAAACCCCTATTCCATGCTGT R: TCCCAAAGTTGGTGGGCCAGAA	159	(Song et al., 2010)

LPL	F: TGGACTGGCTGTCACGGGCT R: GCCAGCAGCATGGGCTCCAA	167	(Song et al., 2010)
GAPDH	F: TCCTGCACCACCAACTGCTT R: TGGCAGTGATGGCATGGAC	100	(Tomokiyo et al., 2008)

Abbreviations: *DSPP*, gene encoding dentin sialophosphoprotein; *Runx2*, gene encoding runt-related protein 2; *OPN*, gene encoding osteopontin; *OC*, gene encoding osteocalcin; *BSP*, gene encoding bone sialoprotein; *ALP*, gene encoding alkaline phosphatase; *PPARγ2*, gene encoding peroxisome proliferator-activated receptor γ2; *LPL*, gene encoding lipoprotein lipase, *GAPDH*, gene encoding glyceraldehyde-3-phosphate dehydrogenase.

III. Results

1. Morphologic Characteristics and Proliferation of SHED

The morphologic characteristics of SHED isolated using different methods were established with the aid of light microscopy. e-SHED exhibited a fibroblastic-like morphology. o-SHED and e-SHED had similar general morphologies (Fig. 1A), but e-SHED had slightly more fibroblastic-like morphology than o-SHED. Despite this similarity there appeared to be minimal differences in their proliferation profiles. Proliferation was initially similar in the two cell types, but after 5 days the proliferation appeared to be a slightly higher for e-SHED than for o-SHED (Fig. 1B). However, the difference was not statistically significant (Mann-Whitney U test, $p>0.05$).

2. CFU-F Assay and Mesenchymal Stem-Cell Marker Expression

Both types of SHED exhibited colony-forming ability (Fig. 2A). The number of colony-forming units was significantly higher for e-SHED (49.2±2.6) than for o-SHED (30.0±2.5; Fig. 2B; Mann-Whitney U test, $p<0.05$).

Differences in the expressions of major surface markers were confirmed by flow cytometry analysis (Fig. 2C). Most of the e-SHED and o-SHED expressed CD90 (>99.0%) and CD105 (>99.0%), which are mesenchymal stem cell markers; the expressions of these two mesenchymal stem cell markers did not differ significantly between the two cell types (Mann-Whitney U test, $p>0.05$). CD146 was expressed in a large proportion of both types of SHED (e-SHED, 86.7%; o-SHED, 83.0%). Two types

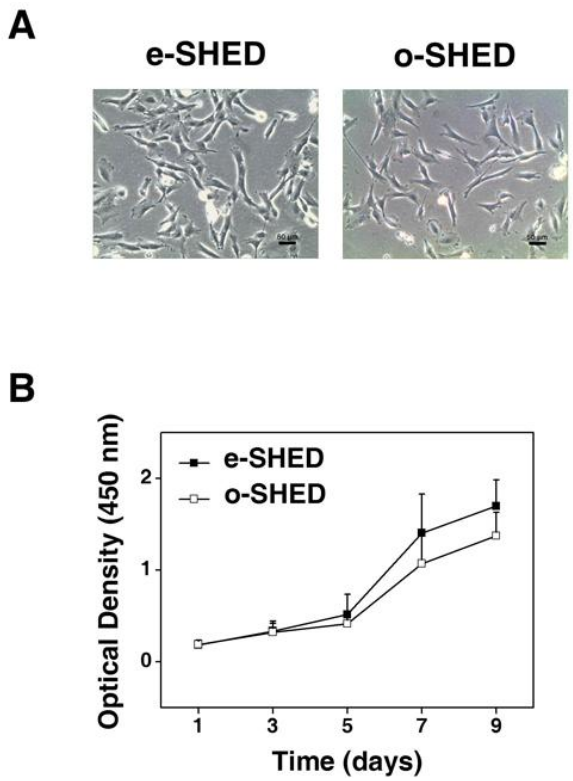


Figure 1. Proliferation of e-SHED and o-SHED. **(A)** Morphologic characteristics. Scale bars: 50 μm. **(B)** Proliferation assay. The *Y*-axis indicates the optical density of yellow-colored formazan. Data were obtained from three independent experiments, with all samples run in triplicate. The data are mean and standard deviation values. The proliferation potential did not differ significantly between the two cell types (Mann-Whitney U test).

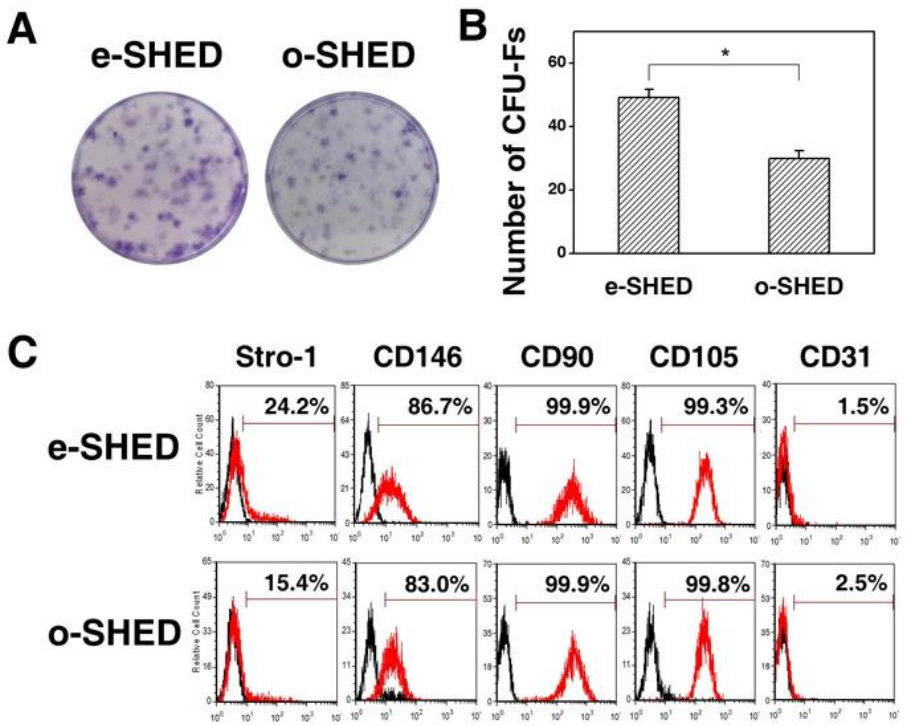


Figure 2. Colony-forming-unit–fibroblast (CFU-F) assay and mesenchymal stem-cell-marker expressions of e-SHED and o-SHED. **(A)** Crystal violet staining. **(B)** The *Y*-axis indicates the number of colonies per 480 cells. Data were obtained from three separate experiments, with all samples run in duplicate. The data are mean and standard deviation values. The colony-forming ability differed significantly between e-SHED and o-SHED (Mann-Whitney U test, $p<0.05$). **(C)** Mesenchymal stem cell marker expression (black lines, controls; red lines, tests). Horizontal bars indicate 1% of control samples. Flow cytometry analysis was performed by three independent experiments.

of cells expressed Stro-1, but the Stro-1 expression of e-SHED (24.2%) was greater than for o-SHED (15.4%). The CD146 and Stro-1 expressions differed significantly between the two SHED types (Mann-Whitney U test, $p=0.05$). However, the expression of CD31 (an endothelial stem-cell marker)—to determine the presence of the endothelial cell contamination—did not differ significantly between the two cell types (e-SHED, 1.5%; o-SHED, 2.5%; Mann-Whitney U test, $p>0.05$). These findings indicate that e-SHED and o-SHED did not contain endothelial cells.

3. *In vitro* Differentiation to Adipogenic and Osteogenic Lineages

Both types of SHED exhibited the ability to differentiate into cells containing lipid vacuoles following treatment with adipogenic induction medium. Oil Red O staining showed that the adipogenic differentiation was greater for e-SHED than for o-SHED (Fig. 3A). The expressions of *PPARγ2* and *LPL*, which are related to adipogenic differentiation, were significantly higher in e-SHED than in o-SHED (as assessed by qPCR; Fig. 3B; Mann-Whitney U test, $p<0.05$).

The two types of SHED also had the ability to differentiate into cells producing mineralized nodules following treatment with osteogenic induction medium. Alizarin Red S staining was conducted to confirm the occurrence of osteogenic differentiation. Although this staining appeared to be slightly higher for o-SHED than for e-SHED, the difference was no statistically significant (Fig. 4A; Mann-Whitney U test, $p>0.05$). Two genes were chosen as markers of osteoblastic differentiation: *ALP* and *BSP*. The

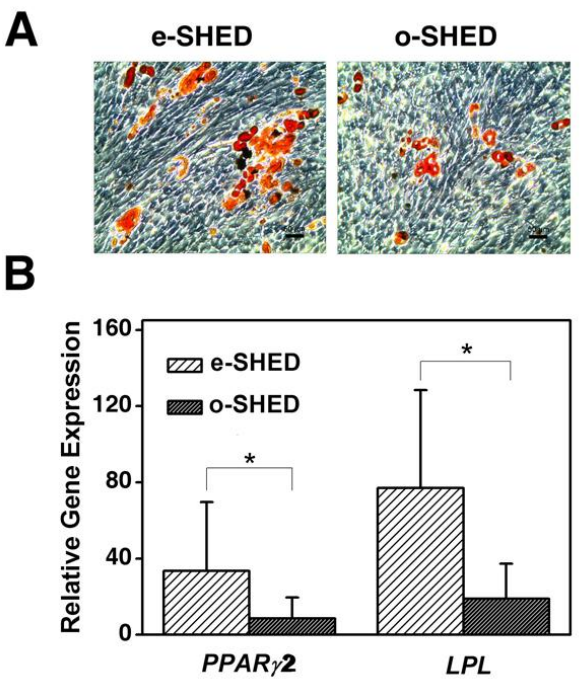


Figure 3. Adipogenic differentiation of e-SHED and o-SHED. **(A)** Oil Red O staining. Data were obtained from five separate experiments, with all samples run in triplicate. Scale bars: 50 μm. **(B)** Changes in the expressions of the genes encoding peroxisome proliferator-activated receptor γ2 (*PPARγ2*) and lipoprotein lipase (*LPL*) after 3 weeks of culture in adipogenic differentiation medium relative to control medium (normalized to 1). Data were obtained from five separate experiments, with all samples run in duplicate. The data are mean and standard deviation values. The expressions of both genes differed significantly between the two cell types (Mann-Whitney U test, $p<0.05$).

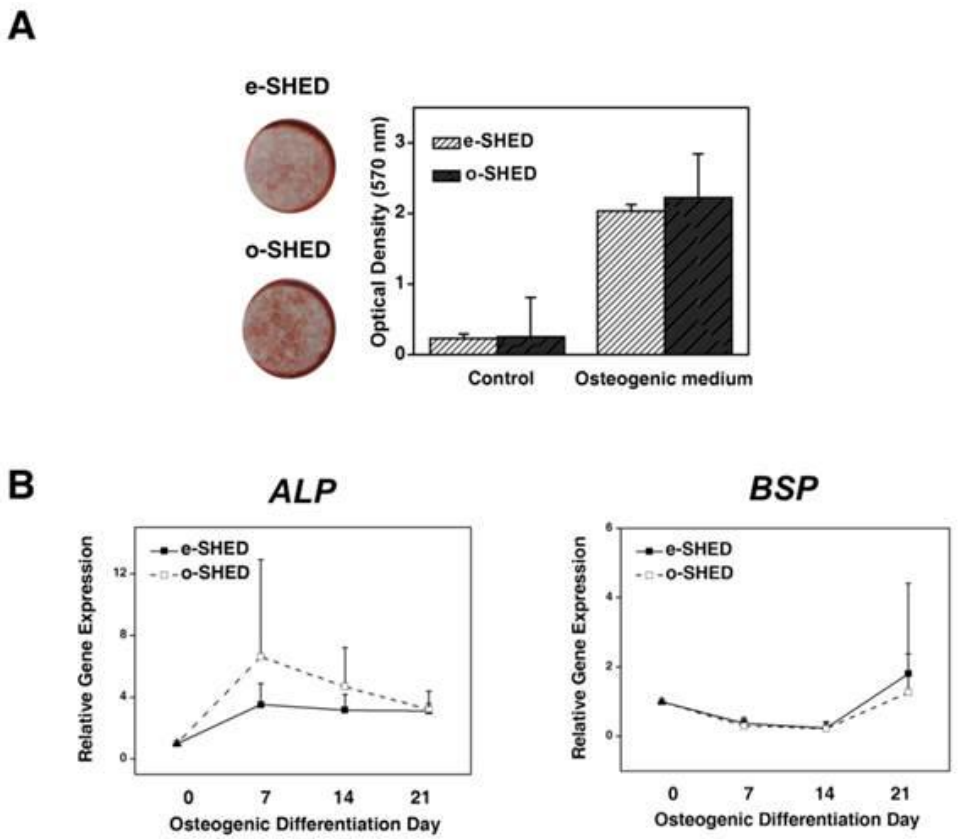


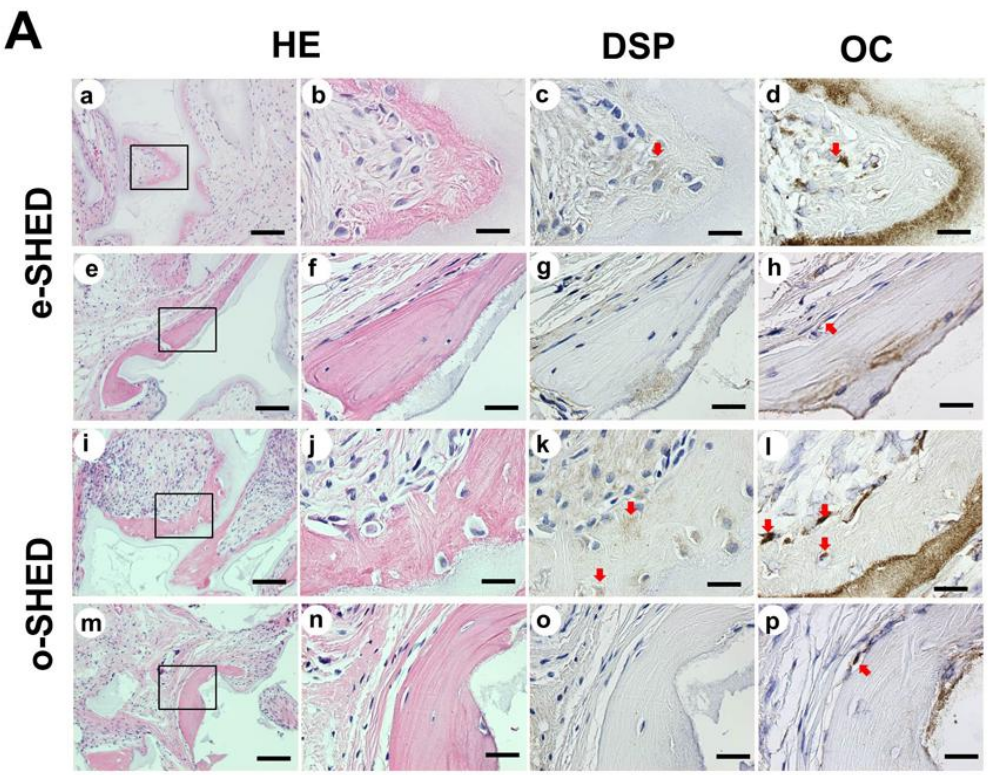
Figure 4. Osteogenic differentiation of e-SHED and o-SHED. **(A)** Alizarin Red S staining and its quantification. Data were obtained from six independent experiments, with all samples run in triplicate. The data are mean and standard deviation values. **(B)** Changes in the expressions of genes encoding alkaline phosphatase (*ALP*) and bone sialoprotein (*BSP*) during osteogenic differentiation. Data were obtained from four independent experiments, with all samples run in duplicate. The data are mean and standard deviation values. The dye accumulation data and gene expression levels did not differ significantly between the e-SHED and o-SHED conditions (Mann-Whitney U test).

expressions of these genes were confirmed by qPCR. The two cell types exhibited a similar expression pattern at specific time points (i.e., at days 7, 14, and 21; Fig. 4B). *ALP* expression increased at the early stage of differentiation and was expressed consistently thereafter, while *BSP* expression decreased slightly until day 14, but then had increased by day 21. There were no significant differences at specific time points (Mann-Whitney U test, $p>0.05$).

4. *In vivo* Transplantation

Both types of SHED could produce hard tissue at the periphery of the MBCP at 9 weeks after transplantation. Significantly more newly formed hard tissues were produced in the o-SHED group than in the e-SHED group (Fig. 5B; Mann-Whitney U test, $p<0.05$). Both the e-SHED and o-SHED produced two types of hard tissues: dentin-like and bone-like (Fig. 5A). The dentin-like hard tissue was characterized by a more linear alignment of the mineral matrix, perpendicular to the lining cell layer, and the absence of embedded cells (Fig. 5A a–d, i–l), while the bone-like hard tissue featured a compact lamellar matrix with cells embedded within it that had reduced cytoplasm and condensed nuclei (Fig. 5A e–h, m–p). Immunohistochemical staining was performed to further characterize the newly formed hard tissues. DSP was only expressed in the dentin-like hard tissues, and OC expression was detected in all samples containing mineralized hard tissues (Fig. 5A).

Figure 6 shows the expressions of gene markers of osteoblast differentiation: *Runx2*, *OC*, *OPN*, and *DSP*. The expressions of all osteoblast-differentiation-related genes


A
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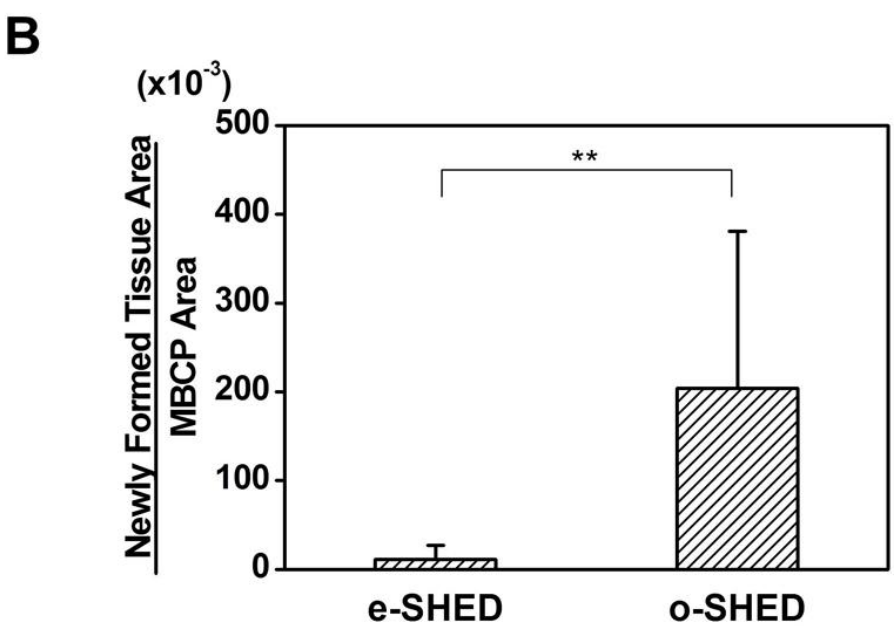
B
(x10-3)
Newly Formed Tissue Area
MBCP Area
500
400
300
200
100
0
**
e-SHED
o-SHED

Figure 5. Comparison of morphologic and immunohistochemical characteristics, and quantitative analysis of the transplants. **(A)** Hematoxylin and eosin (HE) analysis and immunohistochemical staining for dentin sialoprotein (DSP) and osteocalcin (OC) in the transplants. HE and immunohistochemical staining revealed dentin-like structures in both the e-SHED and o-SEHD transplants (a–d, i-l). HE and immunohistochemical staining indicate bone-like structures in both the e-SHED and o-SHED transplants (e-h, m–p). The boxed areas indicated in the photomicrographs in the first column are shown at higher magnification in the other three columns. Scale bars: 100 μm in a, e, i, and m, and 25 μm in b–d, f–h, j–l, and n–p. **(B)** Area of newly formed hard tissue in e-SHED and o-SHED. The *Y*-axis indicates the percent ratio of newly formed tissue area to the macroporous biphasic calcium phosphate (MBCP) particle area. The data are mean and standard deviation values. The area of newly formed hard tissue differed significantly between e-SHED and o-SHED (Mann-Whitney U test, $p<0.05$; $n=19$ for e-SHED and $n=20$ for o-SHED).

appeared to be higher in o-SHED than in e-SHED, but the difference was not statistically significant (Mann-Whitney U test, $p>0.05$). This finding was confirmed by the HE and immunohistochemical staining results. However, ALP analysis revealed that the amount of ALP was significantly greater in o-SHED than in e-SHED (Fig. 6B; Mann-Whitney U test, $p<0.05$).

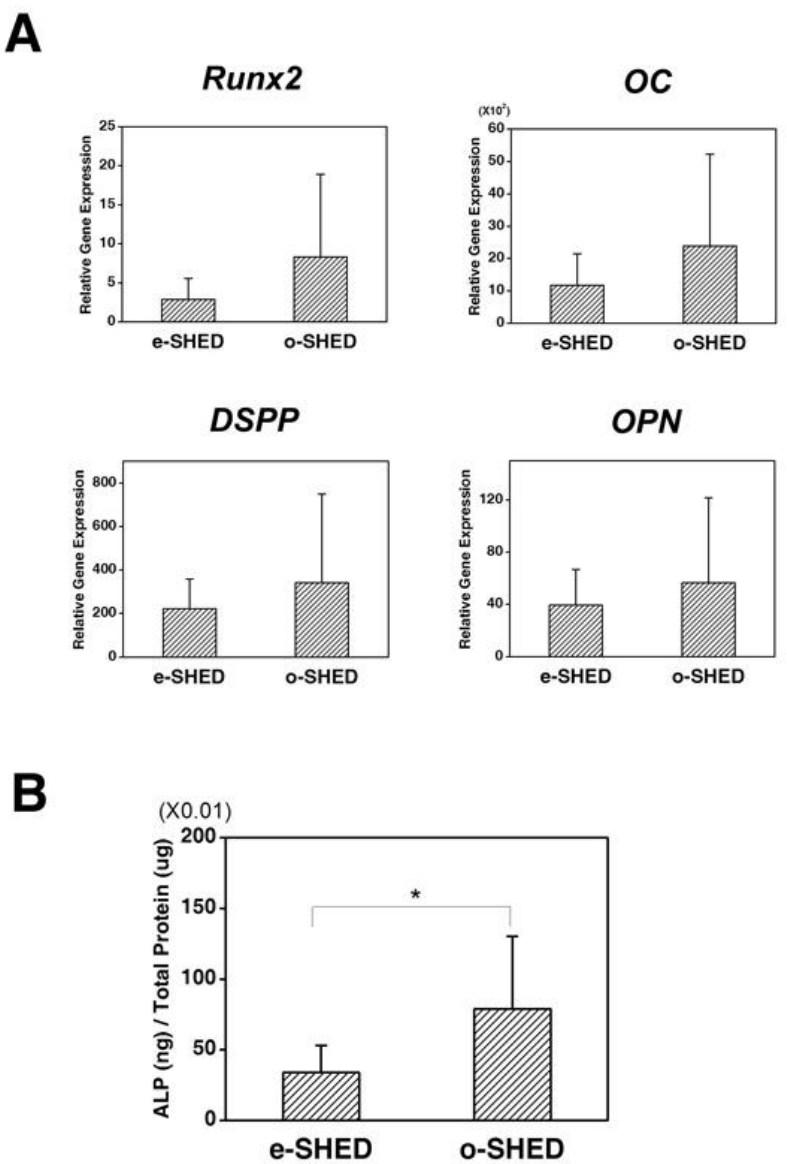

Figure 6. Gene expressions of markers of osteogenic differentiation and ALP activity in the e-SHED and o-SHED transplants. **(A)** Relative expressions of the genes encoding runt-related protein 2 (*Runx2*), OC (*OC*), dentin sialophosphoprotein (*DSPP*), and

osteopontin (*OPN*). The gene expression level of MBCP transplants was set as the control (normalized to 1). Data were obtained from 12 transplant fragments for e-SHED and 18 for o-SHED. The data are mean and standard deviation values. **(B)** Relative quantity of active ALP protein to the total proteins in the transplants. Data were obtained from 20 transplant fragments for e-SHED and 20 for o-SHED. The data are mean and standard deviation values. The differences in gene expressions between the e-SHED and o-SHED conditions were not statistically significant; however, the ALP protein expression did differ significantly between the two cell types (Mann-Whitney U test, $p<0.05$).

IV. Discussion

Stem cells were isolated from human deciduous teeth (SHED) using both the enzymatic disaggregation and outgrowth methods, and the characteristics of each type of SHED (i.e., e-SHED and o-SHED) were confirmed both *in vitro* and *in vivo*. It was found that the colony-forming ability and Stro-1 expression were higher in e-SHED than in o-SHED (Fig. 2), but that the *in vivo* hard-tissue-forming ability was superior for o-SHED than for e-SHED (Figs. 5 and 6).

In the present study, the e-SHED possessed superior colony-forming potential (Fig. 2) and mesenchymal stem cell characteristics—such as Stro-1 expression (Fig. 2) and adipogenic differentiation potential (Fig. 3)—compared with o-SHED. It was suggested that deciduous teeth stem cells have similar growth characteristics, regardless of how they are isolated, but that the expression of the mesenchymal stem-cell marker Stro-1 is higher in enzymatically isolated cells (Bakopoulou et al., 2011). Similarly, although *in vitro* osteogenic differentiation potential was similar in enzymatically and outgrowth-isolated cells, DSPP expression occurred earlier in the former (Bakopoulou et al., 2011). These findings coincide with those of the present study, in which the mesenchymal stem cell characteristics were superior in e-SHED than in o-SHED (Figs. 2 and 3); however, contrary to previous findings, *in vivo* hard-tissue formation was excellent in o-SHED (Figs. 5 and 6).

It is possible that e-SHED can be isolated from either the center of the pulp tissue or from the perivascular sheath region. However, in the present study, Stro-1 expression

was significantly stronger in e-SHED than in o-SHED (Fig. 2). Stro-1 expression is affected by the existence of stem cells. Stro-1 is expressed in the perivascular and perineural sheath region in teeth (Shi and Gronthos, 2003; Kaneko et al., 2009; Song et al., 2010). A small artery and a nerve are located at the center of the pulp, and a capillary and nerve branches are stretched out from the core small artery and nerve trunk (Dahl and Major, 1973; Shi and Gronthos, 2003; Ten Cate, 2008). Therefore, Stro-1 expression is higher at the center of the pulp around the small artery and nerve, and decreases toward the edge. SHED that are isolated from tissues that have been penetrated using an enzyme solution (i.e., the enzyme dissociation method), to produce e-SHED, are more likely to be obtained from the center of the pulp or near blood vessels, which strongly express Stro-1. However, with the outgrowth method, cells are encouraged to grow out from the tissue. The migration time is shorter at the edge of the tissue than at the center, and so SHED isolated using this method, o-SHED, are more likely to be isolated from the margin than the center of the pulp tissue, even if chopped pulp tissues are used for both isolation methods. Therefore, it was inferred that in the present study e-SHED were more likely to have been isolated from the center of the pulp tissue or perivascular sheath than were o-SHED.

The e-SHED exhibited stronger stemness characteristics than o-SHED. Stem cells are able to differentiate into multiple lineages, but progenitor cells are more restricted in their differentiation potency, and differentiate into only one or two lineages. The present flow cytometry results revealed no difference in the expressions of mesenchymal stromal-cell (CD90 and CD105), and endothelial-cell (CD31) markers, but Stro-1 and

perivascular cell (CD146) expression were higher in e-SHED than in o-SHED (Fig 2). Stro-1 is a stem cell marker, and hence a higher Stro-1 expression reflects a higher colony-forming ability and multipotency (Simmons and Torok-Storb, 1991). The present findings confirmed that e-SHED exhibited greater Stro-1 expression, colony-forming ability, and adipogenic differentiation than o-SHED (Figs. 2 and 3).

The o-SHED might be more differentiated cells, and hence more likely to have differentiated into hard-tissue-forming cells than e-SHED. Stem cells interact with the niche within their local microenvironment to maintain their identity, self-renewal, and differentiation potential.1 Stem cells divide asymmetrically, so that one daughter cell stays connected to niche and retains its stem cell identity, while the other daughter moves away from the niche and begins to differentiate. This event is associated with spatial relationships and signals secreted from the niche; stem cells begin to differentiate when they no longer receive signals from the niche or when they move away from it (Hardy et al., 1979; Gonczy and DiNardo, 1996; Yamashita et al., 2003; Jones and Wagers, 2008). The stem cell niche has a higher Stro-1 expression, but Stro-1 expression was weaker in o-SHED than in e-SHED. o-SHED, located in the margin of the pulp, may thus begin to differentiate because it is difficult for them to receive signals secreted from the niche. Furthermore, the o-SHED may have been isolated at a later passage than the e-SHED, and so are more likely to have been further differentiated. Patel et al. isolated cells from mouse pulp tissue and cultured them, and found that the cells became more differentiated at latter passages, as evidenced by measurement of the expressions of stem cell and osteogenic markers using semiquantitative RT-PCR (Patel et al., 2009). o-SHED thus

need more time to reach a similar level of confluence as e-SHED at the first primary culture because of the time required for the cells to grow out from the pulp tissue. e-SHED, obtained via enzyme-induced dissociation, require a relatively short time to reach a particular level of confluence. Therefore, we assumed that in the present study the o-SHED would have been isolated at a slightly later passage than the e-SHED, even after the same number of subcultures, and as such they contained more differentiated cells than e-SHED.

The characteristics of the newly formed hard tissue did not differ between e-SHED and o-SHED. o-SHED produced a large quantity of newly formed hard tissue, had stronger expressions of DSP and OC, and stronger staining for ALP (Figs. 5 and 6). Nevertheless, immunohistochemistry analysis revealed that the expressions of DSP and OC were similar in the two types of SHED. In fact, the two types of newly formed hard tissues exhibited dentin-like and bone-like appearances. This finding concurs with the results of Miura, et al. 2003 (Miura et al., 2003). Thus, there was no difference in the characteristics of the newly formed hard tissues produced by the two types of SHED.

Each of the two methods used to obtain SHED used in this study, enzymatic disaggregation and outgrowth, has advantages and disadvantages. Cells with a greater colony-forming ability are obtained via enzymatic disaggregation when the enzyme is treated at 4℃ for a long period (Engelholm et al., 1985). Furthermore, enzymatically disaggregated cells are capable of multilineage differentiation (Miura et al., 2003; Bakopoulou et al., 2011). However, enzymatic disaggregation is associated with cytotoxicity and is more expensive than the outgrowth method. While it is less expensive,

the outgrowth method is more time consuming, and the proliferation of the cells it yields is slower than those obtained through enzymatic disaggregation (Huang et al., 2006; Tanaka et al., 2011). In addition, outgrowth-isolated cells have lower stem-cell-marker expression than enzymatic disaggregated cells, and thus have lower multilineage differentiation potential (Bakopoulou et al., 2011; Tanaka et al., 2011).

One limitation of the present study is that different pulp tissues were used for the enzymatic disaggregation and outgrowth protocols. While it would have been better to use the same pulp tissues for the two isolation methods, much less pulp tissue is generally available from deciduous teeth than from permanent teeth. It was thus difficult to isolate SHED from the same pulp tissue using two isolation methods. As a compromise, all of the SHED obtained from seven children were pooled in order to reduce the degree of sampling bias in the results for each individual. Furthermore, SHED obtained from similar conditions was assigned to two groups.

This study has clarified the *in vitro* and *in vivo* characteristics of SHED isolated in different ways: enzymatic disaggregation and outgrowth. It was found that the two types of SHED have stem cell characteristics and are capable of differentiating into both adipocytes and osteoblasts. e-SHED can exhibit stronger stemness characteristics, while o-SHED can produce more hard tissues after *in vivo* transplantation. The quality of the newly formed hard tissue produced by e-SHED and o-SHED was similar with respect to morphological characteristics and IHC staining, but on transplantation, the o-SHED produced quantitatively more newly formed hard tissue than e-SHED, presumably because they included more progenitor cells. Therefore, o-SHED might be more suitable

for hard-tissue regeneration therapy in teeth than e-SHED in point of similar quality but a greater quantity of newly formed hard tissue.

V. Conclusion

The objectives of this study were to isolate stem cells from human exfoliated deciduous teeth using enzymatic disaggregation and outgrowth method and investigate the differences in the *in vitro* and *in vivo* characteristics between two types of SHED. Cell proliferation potential, colony-forming ability, stro-1 and CD146 expression, and adipogenic differentiation potential were higher in e-SHED than o-SHED. Although *in vivo* hard tissue formation was greater in o-SHED transplants, there was no difference in the quality of hard tissue generated by e-SHED and o-SHED. In conclusion, e-SHED exhibited stronger stemness characteristics, but o-SHED were more suitable for hard tissue regeneration therapy in teeth.

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국문요약

분리방법에 따른 유치 줄기세포의 특성

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자연탈락 유치에서 얻은 줄기세포는 조직재생치료에 있어서 중요한 치아조직 원천으로 다른 일차배양 방법을 사용하여 얻을 수 있다. 이 연구의 목적은 enzymatic disaggregation과 outgrowth 방법을 사용하여 분리된 유치 치수 줄기세포의 시험관 내와 생체 내에서의 차이를 확인하는 것이다.

유치 치수 줄기세포는 enzymatic disaggregation (e-SHED; 7개)과 outgrowth (o-SHED; 7개) 방법에 의해 14개의 자연탈락 유치로부터 분리되었다. 유치 치수 줄기세포의 증식능, 집락형성능, 간엽성 줄기세포 표식인자의 확인이 시험관 내에서 확인되었으며, 시험관 내 분화가 정량적 PCR과 조직화학적 염색법을 이용해 확인되었다. 또한 생체 내 이식 후 생성된 경조직의 정량적, 정성적 특징은 HE 염색법과 조직화학적 염색법, 정량적 PCR과 정량적인 알칼리성 인산 가수분해효소의 분석을 통해서 확인되었다.

세포 증식능, 집락형성능, stro-1과 CD146의 발현은 o-SHED보다 e-SHED에서 높게 나타났다. 시험관 내 지방분화능은 e-SHED에서 높게 나타난 반면 시험관 내 골분화능은 두 종류의 유치 치수 줄기세포에서 차이가 없었다. 생체 내 경조직 형성은 o-SHED를 이식한 쥐에서 높게 나타났으나 각각의 유치 치수 줄기세포를 이식하여 형성된 경조직의 질적인 차이는 없었다.

본 연구 결과 e-SHED는 높은 줄기세포성을 나타내는 반면 o-SHED는 치아에서 경조직 재생치료에 더 적합할 것으로 보인다.

핵심되는 말: 유치 치수 줄기세포 (SHED), enzymatic disaggregation, outgrowth, 생체 내 이식, 경조직